Medial collateral knee ligament healing

Combined medial collateral and anterior cruciate ligament injuries studied in rabbits

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We examined the histological appearance and biochemical properties of the healing medial collateral ligament (MCL) of a rabbit knee after combined MCL and anterior cruciate ligament (ACL) injury treated with ACL reconstruction and with or without MCL repair. By so doing, we hoped to understand better our previous biomechanical observations (Ohno et al. 1995) and possibly learn where to focus future investigation into improving the quality of the healing MCL.

Ligaments were examined at 6 and 12 weeks of healing. We found healing of all ligaments with hypercellularity and fibroblast elongation along the axis of loading, as expected. Unexpected, however, was the finding of multiple osteophytes in both the repaired and nonrepaired specimens at the medial borders of the joint and at the MCL insertions. These were felt to affect possibly the biomechanics of the MCL by causing stress risers at the point where they undermine the ligament. Biochemically, we demonstrated a correlation between collagen content and hydroxypyridinium crosslinks and modulus of elasticity. While this implies that the modulus is dependent on collagen content and hydroxypyridinium crosslink density, modulus is also probably dependent on other factors such as collagen organization, type and internal structure. Overall, the detailed characterization and correlation between the histological, biochemical, and biomechanical properties of the healing MCL in the severe knee injury model provide insight into the functional behavior of the healing MCL.

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Experimental (Inoue et al. 1990, Weiss et al. 1991) and clinical studies (Sandberg et al. 1987, Ballmer and Jakob 1988, Indelicato 1995) have indicated that an isolated MCL rupture heals well without surgical repair. However, less is known about the appropriate treatment and healing of the MCL when it is involved in more severe knee-ligament trauma, such as a combined injury of the MCL and anterior cruciate ligament (ACL) (Shelbourne and Porter 1992). Generally, ACL reconstruction is recommended, whereas the need for surgical repair of the MCL after such injuries is debated (Warren and Marshall 1978, Shelbourne and Baele 1988, Zarins and Adams 1988, Shelbourne and Porter 1992, Robins et al. 1993). Animal models to study such multiple ligament injuries have been developed in our research center (Ohno et al. 1995) and by others (Bray et al. 1992). Our previous studies indicate that MCL repair after ACL reconstruction leads to less varus-valgus knee laxity and a greater ultimate load than after nonreparative treatment of the MCL. Despite improvements in the structural properties of the femur-MCL-tibial complex (FMTC) and in the mechanical properties of the healing MCL with the repair of the MCL, these experimental values still failed to approach those of their contralateral sham-operated controls (Ohno et al. 1995).

During the healing process, histological and biochemical events occur that correspond to the structural and biomechanical changes we have seen in the MCL. Histologically, 1 week after an isolated strained (grade II) injury of the MCL (Laws and Walton 1988), hypercellular bands of tissue were present adjacent to normal-appearing tissue. An increase in the number of blood vessels and collagen fibers accompanied continued hypercellularity at 3 weeks, with the collagen fibers becoming oriented along the longitudinal axis of the MCL within 6 weeks. Biochemically (Frank et al. 1983, 1995, Weiss et al. 1991, Frank et al. 1994) the early inflammatory phase of healing was characterized by increased water and glycosaminoglycan (GAG) contents, while the long-term remodeling phase corresponded to altered percentages (compared to normal) of certain collagen types (nota-

bly types I and III), as well as continued changes in GAG and collagen content.

We investigated the histological appearance and biochemical properties of the healing MCL after a combined MCL+ACL injury. This information would help to explain the mechanical properties of the healing MCL previously observed by our research center (Ohno et al. 1995). We hypothesized that histomorphological and chemical data (i.e., collagen content and the level of mature collagen crosslinking residues) would correlate with the mechanical observations made previously by Ohno et al. (1995) with regard to the MCL in the MCL+ACL injury. We expect these findings to be similar to the correlations recently noted by Frank et al. (1995) between collagen content and tensile strength and between crosslinking density and tensile strength in the isolated rabbit MCL injury.

Animals and methods

In the left knee of 32 skeletally mature New Zealand white rabbits (weight 4.1 ± 0.4 kg), a lateral parapatellar incision was created and reflected to access the MCL. The MCL substance was ruptured in a mop-end fashion by pulling medially on a rod passed beneath the ligament, as described elsewhere (Weiss et al. 1991). The ACL was transected at its tibial insertion and the ligament tissue removed. As described by Ohno et al. (1995), the ACL was reconstructed using a flexor tendon allograft which was trimmed to approximately 4 mm in width and 6 cm in length at the time of surgery. A custom-made, 8-mm diameter stainless-steel button was secured to one end of the graft with two 2-0 nylon sutures. The graft was passed through a 3-mm wide tibial tunnel into the knee joint and then placed over the top of the lateral femoral condyle. A 10-N force was applied to the graft while it was secured to the lateral femoral cortex with a titanium staple (7 × 10 mm) and to the periosteum with four 3-0 braided nylon sutures. In the right knee, the MCL was exposed and undermined to provide a sham-operated control.

The repair group comprised 16 rabbits, in which the ruptured MCL was repaired, using a modified Kessler technique (Weiss et al. 1991). In the remaining 16 rabbits (the nonrepair group), the ends of the ruptured MCL were apposed, but not repaired. All wounds were closed routinely. The rabbits were allowed cage activity, food and water *ad libitum*. 8 rabbits from the repair group and 8 from the nonrepair group were killed 6 weeks postoperatively; the remaining 8 in each group were killed 12 weeks postoperatively.

The hind limbs of 2 rabbits in each group were harvested for histological analysis. The musculature and joint capsule were removed from the knee joints so that a sample of MCL midsubstance could be obtained by a transverse cut across the ligament. The femoral and tibial insertions were removed from the bone, using an autopsy saw such that a small section of bone remained attached to the ligament ends. These specimens contained samples of bone and marrow, articular cartilage of the medial compartment, as well as ligament. All tissues were fixed for a minimum of 1 week in 10% neutral buffered formalin.

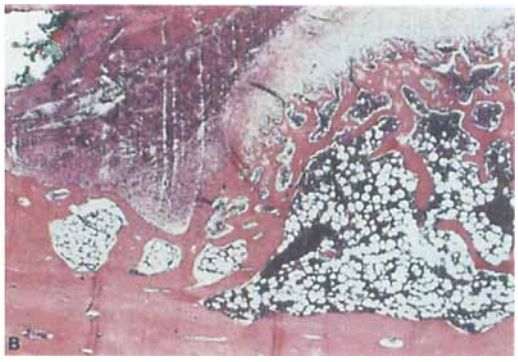
Both the midsubstance and insertions of each MCL were bisected in the midcoronal plane (i.e., along the long axis of the MCL, midway between its anterior and posterior margins). These samples were then demineralized in 8% formic acid and dehydrated in an ethanol series, infiltrated in a solution of 2% Necoloidine solution for 4 days, cleared briefly in chloroform, and embedded in paraffin (Bancroft and Stevens 1982). This technique, known as double-embedding, allows for uniform sectioning through ligament into bone. Sections 6 micrometers thick were cut with a rotary microtome and a steel blade. These sections were stained with hematoxylin and eosin, Goldner's trichrome or toluidine blue O, and were examined by light and polarized light microscopy. The ligament cellularity, collagen organization, matrix metachromasia and vascularity were assessed qualitatively, as also were organization of the bone, marrow and articular cartilage. These assessments were done in a blinded, randomized fashion.

6 of the 8 rabbits in each group were used for biochemical evaluation following biomechanical testing (Ohno et al. 1995). Approximately 1 cm of substance tissue was dissected from the center of each MCL. After being washed in cold distilled water, these samples were lyophilized for 18 hours to determine the dry weight, which ranged between 3.0 and 5.0 mg, depending on the cross-sectional area of the healing ligament in the central region. Tissues were then extracted in 1M guanidinium hydrochloride for 24 hours, after which they were washed again, lyophilized and reweighed. Samples were then hydrolyzed in 6M HCl at 108 °C for 18 hours. The hydrolysates were neutralized by dilution in water and dried on a concentrator. The dried samples were reconstituted in 1% n-heptafluorobutyric acid. Small aliquots of these samples were taken for hydroxyproline determination of collagen content (Woessner Jr. 1961) and for analysis of mature hydroxypyridinium crosslinking residues (Eyre et al. 1984).

Briefly, for collagen content determination, after hydroxyproline oxidation with sodium p-toluene-

Figure 1. 2 MCL femoral insertion sites after 12 weeks of healing, HE ×40.

Control specimen.



Experimental specimen.

sulfonchloramide and subsequent reduction with p-dimethylaminobenzaldehyde, samples were incubated at 60 °C. Triplicate 200-µm aliquots of the samples were placed in 96 well plates and read spectrophotometrically at 550 nm. The collagen content was estimated by assuming that that 13% of the amino acid composition of collagen is hydroxyproline.

For crosslinking analysis, the hydroxypyridinium crosslinking residues were resolved on a reverse-phase C18 column using a gradient of acetonitrile in water and 0.01% n-heptafluorobutyric acid as an ion-pairing agent (Eyre et al. 1984). The hydroxypyridinium residues were delivered using a pure hydroxypyridinoline standard of known concentration.

Paired Student's t-tests were used to compare differences in biochemical properties between the sham-operated control specimens and the repaired or non-repaired knees. Two-way analysis of variance with post-hoc testing was used to assess the effects of postoperative healing time and treatment method on the biochemical properties and to detect interactions between time and treatment. Linear and multiple regression and correlation analyses were performed to determine how well the mechanical properties of the healed MCL (Ohno et al. 1995) correlated with both collagen content and hydroxypyridinium crosslinks. The significance level for all statistical tests was set at $p < 0.05$.

Results

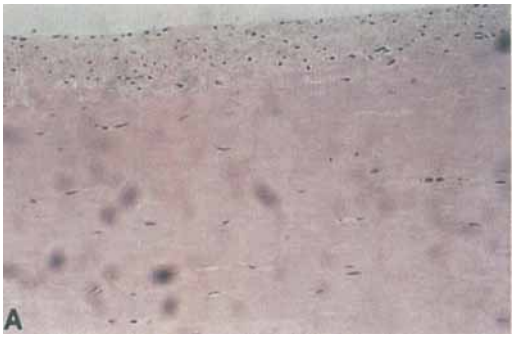
Gross inspection of the knees revealed that all experimental MCLs had healed. Specifically, translucent material had formed at the rupture site of the repaired and nonrepaired MCLs; the transition between this tissue and the original tissue was barely discernible. Osteophytes had formed at the medial aspect of the medial femoral condyle of all experimental (repaired and non-repaired) knees by 6 weeks, with osteophyte growth on the medial tibial plateau in some cases. Osteophytes were also present at the lateral periphery of the patellofemoral joint. Within 12 weeks, the osteophytes had increased in both size and number, sometimes extending to the lateral periphery of the tibial plateau. The marrow within all osteophytes, whether from a repaired or nonrepaired MCL, was hypercellular (Figure 1).

In contrast to the osteophyte formation, the organization and quality of the articular cartilage in the medial compartment seemed largely unaffected by the mop-end injury. The articular cartilage was slightly hypercellular in only three of the 12-week experimental specimens.

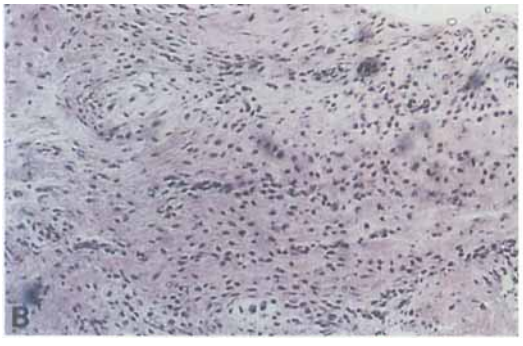
The ligament at both the femoral and tibial insertion sites tended to be hypercellular and hypervascular, regardless of whether the MCL had been repaired (Figure 1). At 6 weeks, the midsubstances of both the repaired and nonrepaired MCLs were hypercellular and the tissue characterized by randomly-arranged immature fibroblasts with large, round nuclei. There was no discernible difference between the repaired and nonrepaired MCLs. After 12 weeks of healing, the midsubstance remained hypercellular, although less so than at 6 weeks. Compared to the 6-week healing tissue, the fibroblast nuclei of the 12-week healing tissue were more elongated and were beginning to align along the long axis of the ligament (Figure 2). As at 6 weeks, there was no discernible difference between the repaired and nonrepaired MCLs.

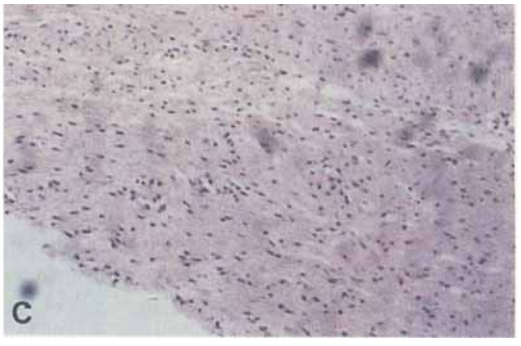
In contrast to the experimental ligaments, the control MCLs were shiny, white and opaque. No osteophytes had formed at the joint surfaces by 6 weeks. At 12 weeks, however, gross osteophytes had formed on the medial surface of the medial femoral condyle in three control joints contralateral to the repaired MCLs

Figure 2. Samples of MCL midsubstance, HE ×200.

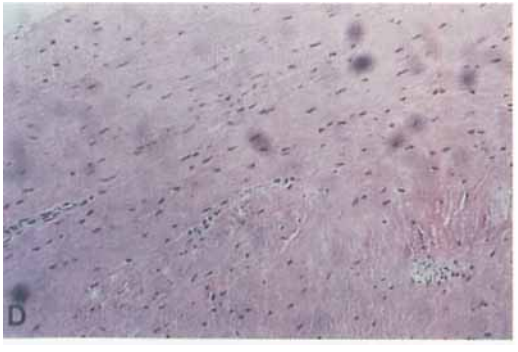


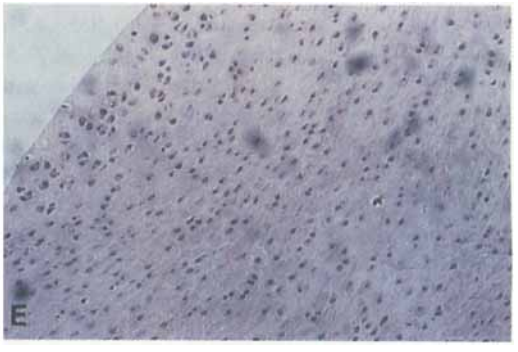
A. Control specimen after 6 weeks of healing.





B, C. Nonrepaired and repaired experimental specimens after 6 weeks of healing.





D, E. Nonrepaired and repaired experimental specimens after 12 weeks of healing.

and in two control joints contralateral to the nonrepaired MCLs. In each case, patellar dislocation was noted during joint dissection, but only one more case of patellar dislocation was noted overall. Nonetheless, for the control MCLs, all histologic qualities were assessed, including cellularity, collagen organization, matrix metachromasia and vascularity of ligaments; and organization of bone, marrow and articular cartilage were within normal limits. One of the four 12-week control MCLs had histologic osteophyte formation near both the femoral and tibial insertions, with the marrow present in the osteophyte and the epiligament being hypercellular.

The collagen content of the 6- and 12-week specimens are shown in Table. We found a significant difference only between the 6-week repair and sham groups. The 6-week nonrepair group and the 12-week repair and nonrepair groups did not differ significantly from their contralateral controls. Neither treatment method nor healing time had a significant effect on collagen content.

At 6 weeks, the density of hydroxypyridinium (hp) crosslinks in both the repaired and nonrepaired MCLs was far lower than that of their respective control

Biochemical properties of the healing MCL at 6 and 12 weeks. Mean (SD)

	6 weeks		12 weeks	
	Control (n 6)	Experimental (n 6)	Control (n 6)	Experimental (n 6)
Repair group				
Collagen content (%)	88 (7.9)	72 (5.5) [a]	83 (11)	73 (15)
Crosslinking (mol hp/mol collagen)	0.85 (0.14)	0.35 (0.11) [a]	0.69 (0.18)	0.54 (0.16)
Nonrepair group				
Collagen content (%)	85 (12)	74 (12)	87 (9.6)	81 (15)
Crosslinking (mol hp/mol collagen)	0.66 (0.36)	0.39 (0.20) [a]	0.82 (0.17)	0.73 (0.22)

[a] Indicates difference ($p < 0.05$) between experimental and control groups.

specimens (Table). Within 12 weeks, these values had increased to 0.54 (SD 0.16) and 0.73 (SD 0.22) mol hp/mol collagen for the repaired and nonrepaired MCLs, respectively, and were no longer significantly lower than their respective controls (Table). Although the treatment method had no significant effect on the density of hydroxypyridinium crosslinks, these crosslinks increased significantly with the healing time. No significant interaction was found between the treatment method and healing time.

Linear regression analyses of collagen content and hydroxypyridinium crosslinks data with respect to the modulus of elasticity were performed with both time periods and repair and nonrepair groups taken together. We found that neither biochemical parameter had a significant correlation with the modulus when considered individually ($r^2 < 0.01$, $p = 0.8$ for collagen content; $r^2 = 0.11$, $p = 0.2$ for hydroxypyridinium crosslinking). However, when collagen content and hydroxypyridinium crosslinks were considered together using multiple regression, a significant, albeit weak, correlation was found ($r^2 = 0.20$, $p = 0.02$).

Discussion

We have previously hypothesized that ACL reconstruction after a combined MCL+ACL injury would provide sufficient initial knee stability to promote MCL healing without the need for MCL repair (Engle et al. 1994, Ohno et al. 1995). However, there was a time-dependent increase in knee instability. Such instability was accompanied by the formation and growth of osteophytes in the tibiofemoral joints, as noted on gross examination. In this environment, repair of the ruptured MCL seemed to provide an initial increase in varus-valgus joint stability which resulted in significant increases in the ultimate load on the FMTC.

In this study, we found a widespread formation of large osteophytes at both MCL insertion sites and at the joint periphery. Although reports of peripheral osteophytes after ligamentous injury are not uncommon, the finding of medial osteophytosis at the MCL insertions has not been reported previously. This medial osteophyte formation may be secondary to primary injury at the insertion site from the mop-end tear; due to post-traumatic changes in the *in situ* loading of the MCL which are reflected in changes in the local effects of load on the insertion site; or due to a global response of massive injury to the ACL and MCL. This possibility, however, is difficult to interpret at present.

Whatever its cause, the presence of osteophytes is relevant to the biomechanical properties of the MCL. Their presence between the MCL and medial condyle, especially given their size, increases the length the MCL must traverse between insertions and may act as stress risers at the point where they undermine the ligament. These effects would alter the structural properties of the bone-ligament-bone complex. Moreover, the mere presence of an osteophyte may change the dynamics of the insertion site, thus increasing the likelihood of insertion-site failure.

The healed MCL remained hypercellular throughout the study period, probably reflecting the regenerative response that the cells were undergoing. The fibroblasts of the midsubstance were elongated and aligned with the long axis of the ligament at 12 weeks. While this appearance is commonly thought to be a response to applied stress (Matyas et al. 1995), previous research has suggested that alignment of collagen fibers along the functional loading axis may lead to increases in tensile strength (Gomez et al. 1989). Our past and present findings support this, because even though the fiber organization was still abnormal at 12 weeks (Weiss et al. 1991), the modulus increased with time, the change correlating with the early alignment of the fibroblasts and collagen fibers.

The significantly lower collagen content of the 6-week repair group, as compared to its control group and the recovery of near-normal collagen content by 12 weeks in both the repaired and nonrepaired groups is consistent with findings in the literature (Frank et al. 1994). However, the slower return to normal of the modulus of elasticity implies that collagen content alone is not responsible for the mechanical properties of ligament healing. Despite the recovery of near-normal collagen content, altered collagen composition, such as a greater ratio of type III to type I collagen (Gomez et al. 1989), might impair the healing of tissue.

The lower concentrations of hydroxypyridinium crosslinks at 6 weeks than at 12 weeks in the experimental MCLs can be explained by remodeling of the tissue and laying down of new collagen, which is initially crosslinked by borohydride-reducible crosslinking residues. These reducible crosslinks mature with time to nonreducible hydroxypyridinium crosslinking residues. While such a maturation process clearly had time enough to occur, the observation that the hydroxypyridinium crosslinks did not correlate well with modulus also implies that it alone is not responsible for the modulus of elasticity.

The lack of linear correlation of either biochemical parameter with modulus may be because the modulus depends on many factors. This is strongly indicated by our finding that the collagen content and hydroxypyridinium crosslinking considered together weakly but significantly correlated with the modulus of elasticity. The weakness of this correlation is probably explained by the fact that other factors such as collagen organization, collagen type and ligament heterogeneity may also come into play. In the healing state, the collagen content and crosslinking may reach normal levels, while their organization and constitution may not.

Our findings regarding collagen content and hydroxypyridinium crosslinks conflict with results of a study recently reported by Frank et al. (1995). They found a significant correlation between the collagen content and modulus of experimental ligaments and a significantly lower crosslinking density in experimental ligaments than in normal control ligaments, even after 40 weeks of healing. The differences in the outcomes of these two studies may reflect differences in the injury model. Our model of MCL rupture with concomitant damage to the insertion sites resulted in a more diffuse injury along the FMTC, and therefore may have a different healing response.

Thus, the detailed characterization and correlation between the histological, biochemical and mechanical properties of the healing MCL in the severe knee injury model provide insight into the functional behavior of the healing MCL. While these correlations may not completely explain the biomechanical function of the healing MCL, they provide a basis for future investigation into the effects of time and of various treatments on improving its quality.

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